European Journal of Pharmacology 875 (2020) 173021

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Morphine-3-glucuronide causes antinociceptive cross-tolerance to morphine and increases spinal substance P expression

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ARTICLE INFO

Keywords:
Dose-response relationship
Drug tolerance
Opioid
Morphine
Morphine-3-glucuronide
Substance P

ABSTRACT

Morphine-3-glucuronide (M3G), the main metabolite of morphine, has been implicated in the development of tolerance and of opioid-induced hyperalgesia, both limiting the analgesic use of morphine. We evaluated the acute and chronic effects of M3G and morphine as well as development of antinociceptive cross-tolerance between morphine and M3G after intrathecal administration and assessed the expression of pain-associated neurotransmitter substance P in the spinal cord. Sprague-Dawley rats received intrathecal M3G or morphine twice daily for 6 days. Nociception and tactile allodynia were measured with von Frey filaments after acute and chronic treatments. Substance P levels in the dorsal horn of the spinal cord were determined by immunohistochemistry after 4-day treatments. Acute morphine caused antinociception as expected, whereas acute M3G caused tactile allodynia, as did both chronic M3G and morphine. Chronic M3G also induced antinociceptive cross-tolerance to morphine. M3G and morphine increased substance P levels similarly in the nociceptive laminae of the spinal cord. This study shows that chronic intrathecal M3G sensitises animals to mechanical stimulation and elevates substance P levels in the nociceptive laminae of the spinal cord. Chronic M3G also induces antinociceptive cross-tolerance to morphine. Thus, chronic M3G exposure might contribute to morphine-induced tolerance and opioid-induced hyperalgesia.

1. Introduction

Opioids are used to treat moderate-to-severe acute and chronic pain but adverse effects such as respiratory depression, nausea, somnolence, dependence, tolerance and opioid-induced hyperalgesia limit their use (Kalso et al., 2004; Rivat and Ballantyne, 2016; Roeckel et al., 2016). In humans, morphine is mainly metabolised to morphine-3-glucuronide (M3G) and morphine-6-glucuronide by hepatic UDP-glucuronosyltransferase 2B7 (Coffman et al., 1997). However, rats form only M3G (Coffman et al., 1997; Coughtrie et al., 1989). M3G has excitatory effects (Hemstapat et al., 2009; Komatsu et al., 2016; Suzuki et al., 1993) and it has been proposed to be partly responsible for morphine tolerance and opioid-induced hyperalgesia (Ekblom et al., 1993; Smith and Smith, 1995). M3G has low affinity (Mignat et al., 1995) to and may be a partial and biased agonist at the μ receptor (Roeckel et al., 2017). Therefore, a high central nervous system concentration of M3G is needed for a clinically significant μ receptor mediated effect. As a polar molecule, M3G should not cross the blood-brain barrier to a large extent (Bickel et al., 1996). However, in long-term administration, four times higher concentrations of M3G than morphine have been measured in the cerebrospinal fluid of patients receiving high doses of morphine (Goucke et al., 1994). We are not aware of studies that would have assessed the effects of chronic M3G treatment on the development of opioid tolerance and tactile allodynia. Morphine is known to increase substance P levels and activate glial cells (King et al., 2005; Ma et al., 2001; Raghavendra et al., 2002), but the effects of M3G are not known.

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https://doi.org/10.1016/j.ejphar.2020.173021
Received 11 November 2019; Received in revised form 11 February 2020; Accepted 14 February 2020
Available online 26 February 2020

Our aim was to assess whether chronic M3G treatment can sensitise rats to a mechanical stimulus and whether M3G may cause morphine tolerance independent of morphine. We evaluated the development of antinociceptive cross-tolerance between morphine and M3G after intrathecal administration and assessed glial activation and substance P levels in the spinal cord.

2. Materials and methods

2.1. Animals

Experiments were approved by the Southern Finland Regional State Administrative Agency (ESAVI-9697/04.10.07-2017). We followed the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the EU2010/63 legislation with adherence to the ARRIVE guidelines. Adult male Sprague-Dawley rats (180–250 g) were housed in clear plastic cages in temperature- and light-controlled rooms (23 ± 2 °C and light cycle of 12 h). Food and water were available *ad libitum*. Before experiments, the animals were habituated to the experimental environment for 5 days, 2 h daily. After intrathecal catheterisation, the animals were housed individually. After the experiments, animals were deeply anaesthetised and euthanised. The number of animals in experimental groups was kept as small as possible (Zimmermann, 1983), but high enough to reach significant statistical power.

2.2. Drugs

Morphine hydrochloride, ketamine hydrochloride (Ketaminol vet®, Intervet, Boxmeer, Netherlands), lidocaine hydrochloride (Lidocain®, Orion Pharma, Espoo, Finland), and medetomidine hydrochloride (Domitor®, Orion Pharma) were purchased from the University Pharmacy (Helsinki, Finland). Morphine 3-β-*D*-glucuronide was a kind gift from the National Institute of Drug Abuse (Bethesda, MD, USA). Morphine and M3G were dissolved in physiological 0.9% saline. Intrathecal drugs were administered in a volume of 10 µl followed by a flush of 15 µl saline. The choice of the intrathecal test dose of morphine was based on a previous study where acute administration of 1.5 µg of morphine caused significant antinociception (Lilius et al., 2012) but tolerance developed to the same dose after a 4-day exposure to intrathecal morphine. The threefold higher (5 µg intrathecal) dose of M3G was based on our unpublished pilot studies where this dose produced significant nociception and on clinical data (Goucke et al., 1994) where four times higher concentrations of M3G than morphine could be measured from cerebrospinal fluid.

2.3. Intrathecal catheter implantation

Rats were anaesthetised with subcutaneous ketamine (50 mg/kg) and medetomidine (0.4 mg/kg). After verifying the absence of a reaction to paw pinch, the animal was placed on a stereotactic frame. The cisterna magna was surgically exposed as earlier described (Yaksh and Rudy, 1976). An 8-cm 26G intrathecal catheter (0.36 mm outer diameter, 0.18 mm inner diameter, item no. 0007740, Alzet, Durect, Cupertino, CA, USA) was carefully inserted into the subarachnoid space to the lumbar enlargement area level through the cisterna magna. The cannula was sutured to neck muscles and the wound was closed with 5–0 sutures. The catheter was filled with 15 µl of saline and the tip closed by cauterisation. After recovery for 3–4 days, the correct placement of the catheter was confirmed with 15 µl (20 mg/ml) of lidocaine, followed by a flush of 15 µl saline. Only rats showing reversible symmetrical paralysis of the hind limbs were accepted for experiments.

2.4. Behavioural testing

Von Frey testing for tactile allodynia and mechanical nociception was conducted with nine different calibrated monofilaments (2, 4, 6, 8, 10, 15, 26, 60 and 100 g, North Coast Medical, Morgan Hill, CA, USA) in ascending order. The hind paws of the rats standing on a metal mesh covered with a plastic dome were stimulated 5 times with each filament with 1-min intervals. Von Frey data are presented with the percent response method (Deuis et al., 2017) as the sum of withdrawal responses (0–5) and summed up separately for thin filaments (2–15 g), for the assessment of tactile allodynia, and thick (26–100 g) filaments, for assessing nociception. Maximum summed response rate is therefore 30 for thin and 15 for thick filaments. When an animal responded five times out of five to a certain force, the rest of the thicker filaments were registered as eliciting a maximum response for the particular time point.

2.5. Immunohistochemistry

Spinal cord samples were collected from the lumbar enlargement (T13–L5) after transcardial perfusion with 4% paraformaldehyde (PFA). PFA-fixed samples were embedded to paraffin blocks, sectioned to 10 µm slices, and probed with antibodies for ionized calcium-binding adapter molecule 1 (IBA1) (1:1000, Cat N 019–19741, Wako Richmond, VA, USA), glial fibrillary acidic protein (GFAP) (1:400, Cat G-3893, Sigma-Aldrich, St. Louis, MO, USA) or substance P (1:10,000, Cat No. T-4107, Peninsula Laboratories, CA, USA). Secondary antibodies, conjugated with horseradish peroxidase and DAB as a substrate were used to visualise bound primary antibodies (DAB peroxidase substrate kit, Vector Laboratories, CA, USA). Slides were scanned with 3DHISTEC (3DHISTECH, Budapest, Hungary) in the core facility of the University of Helsinki (https://www.fimm.fi/en/services/technology-centre/digital-and-molecular-pathology-unit). Nociceptive laminae (I, II and V) of the spinal cord were cropped with Panoramic Viewer (3DHISTEC) and CorelDraw X8 (Corel Corporation, ON, Canada) using a spinal cord model figure (Paxinos and Watson, 1998). Two samples per animal were analysed and the average used. The number of IBA1 or GFAP positive cells and area of staining of IBA1, GFAP and substance P were quantified with Matlab R2014b (Mathworks, Natick, MA, USA) as previously described (Jokinen et al., 2018).

2.6. Experiment 1: effects of chronic intrathecal morphine and M3G on tactile allodynia and antinociceptive tolerance

On the first day, opioid-naïve rats received either an intrathecal test dose of 1.5 µg morphine or 5 µg M3G (Fig. 1). The treatment was continued twice daily with 12-h intervals with the same drug with the

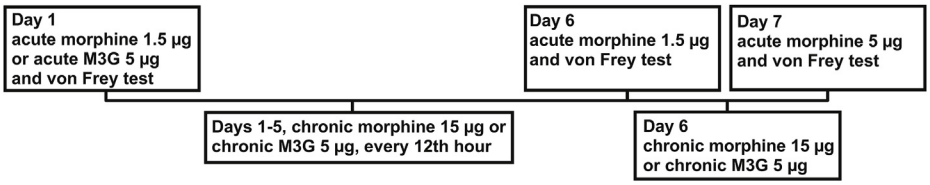

Fig. 1. Intrathecal drug treatment scheme from the first day to the morning of the seventh day in the chronic morphine and chronic morphine-3-glucuronide (M3G) treatment groups. Behavioural testing was conducted in the morning of day 1 after acute morphine (1.5 µg) or M3G (5 µg), in the morning of day 6 after chronic treatments (morphine 15 µg or M3G 5 µg) and after an acute dose of morphine (1.5 µg), and in the morning of day 7 after chronic treatments and acute dose of morphine (5 µg).

exception that the morphine dose was increased to 15 μg. In the morning of day 6, both groups received 1.5 μg of intrathecal morphine to test for possible antinociceptive tolerance and cross-tolerance or hyperalgesia. Twelve h later, animals received their chronic treatment (morphine 15 μg or M3G 5 μg) and in the morning of day 7, both groups received a higher 5 μg dose of morphine. Behavioural testing (von Frey) was conducted on days 1 (acute effects of morphine or M3G), 6 (morphine 1.5 μg), and 7 (morphine 5 μg) before and 30, 60, and 120 min after the test dose.

2.7. Experiment 2: effects of chronic intrathecal morphine and M3G on glial activation and substance P expression in the spinal cord

To assess the possible actions of morphine and M3G on substance P or glial marker expression, a second group of rats was treated with intrathecal M3G (5 μg), morphine (15 μg) or saline twice a day with 12-h intervals. On the fifth day, spinal cord samples were collected and analysed for the expression of IBA1 (microglia), GFAP (astrocytes) and substance P.

2.8. Statistical analysis

Statistical analyses were performed with Graphpad Prism 7 (GraphPad Software, La Jolla, CA, USA). Individual filaments (Fig. 2) are presented as mean with S.E.M. and analysed with the two-way ANOVA followed by Bonferroni's test. Summarised results (Fig. 3 and Fig. 4) are presented as medians with interquartile ranges and analysed with the Friedman test followed by Dunn's correction for multiple comparisons. Immunohistochemistry data are presented as mean of the sample values with S.E.M. and analysed by one-way ANOVA with Holm-Sidak correction for multiple comparisons. The difference was considered significant at $P < 0.05$.

3. Results

3.1. Experiment 1: chronic intrathecal administration of M3G causes antinociceptive cross-tolerance to morphine

On day 1, drug-naïve animals showed only a minor response to the thin (2–15 g) filaments but responded strongly to thick (26–100 g) filaments at the baseline measurements (Fig. 2A) suggesting that the thin filaments can be used to assess allodynia whereas the responses to thick filaments represent nociception. Therefore, we analysed the response rates to thin and thick filament also separately (Fig. 3A–B). On day one, acute morphine (1.5 μg, intrathecal) did not affect the response rate to thin filaments but it decreased the response rate to thick filaments 30 min after administration (Fig. 3A and B). In contrast, acute M3G (5 μg, intrathecal) increased the response rate to thin filaments at 30 min (Fig. 3A). Near-maximum response rate to thick filaments was already achieved in the baseline measurements and M3G did not affect it (Fig. 3B).

After administering intrathecal morphine (15 μg) or M3G (5 μg) twice daily for five days, the baseline values were measured on Day 6, 12 h after the previous drug administration (Fig. 2B). The median response rate to the thin von Frey filaments increased from 2 to 15 responses after chronic morphine administration and from 3 to 13 responses after chronic M3G administration (Fig. 4A). Neither drug affected the baseline response rate to thick filaments as maximum response was already achieved in baseline measurements on Day 1 (Fig. 4B). After baseline measurements, acute morphine (1.5 μg, intrathecal) decreased the response rate to thin filaments, both in the chronic morphine (at 30, 60 and 120 min) and chronic M3G (at 30 and 60 min) groups (Fig. 3C) indicating relief of allodynia. In contrast, response rate to thick filaments showed no significance suggesting development of antinociceptive cross-tolerance and tolerance (Fig. 3D).

On Day 7, the baseline values were at the same level as on Day 6 in both treatment groups (Fig. 4A and B). After administration of a higher dose of morphine (5 μg, intrathecal), a significant decrease of the median response rate to thin filaments was seen at 60 min, and 60 and 120 min (Fig. 3E) and thick filaments at 60 min, and 60 and 120 min in the chronic morphine and M3G treatment groups, respectively (Fig. 3F). Distribution in response rate to different filaments is presented in Fig. 2C.

3.2. Experiment 2: chronic intrathecal morphine and M3G increase the expression of substance P, but not markers of glial activation, in the spinal cord

3.2.1. Substance P

Compared with the saline-treated group, the area covered by substance P positive staining in the dorsal horn laminae I–II was increased by 35% and by 41%, respectively, in the chronic morphine- and the M3G-treated groups (Fig. 5A). In lamina V, only the effect of morphine treatment reached statistical significance compared with the saline group (Fig. 5B). Intrathecal catheterisation itself did not affect substance P expression in any of the analysed laminae (Fig. 5A–B). Representative staining images for each group are shown in Fig. 5C.

3.2.2. IBA1

Compared with the intrathecally catheterised animals that received saline, chronic morphine or M3G treatments did not increase the area of positive staining of the microglial marker IBA1 or the number of positive cells in either spinal cord laminae I–II (Fig. 6A and C) or lamina V (Fig. 6B and D). However, intrathecal catheterisation and saline treatment itself increased the number and the area of IBA1-positive cells (Fig. 6A and C) in laminae I–II. Representative staining images for each

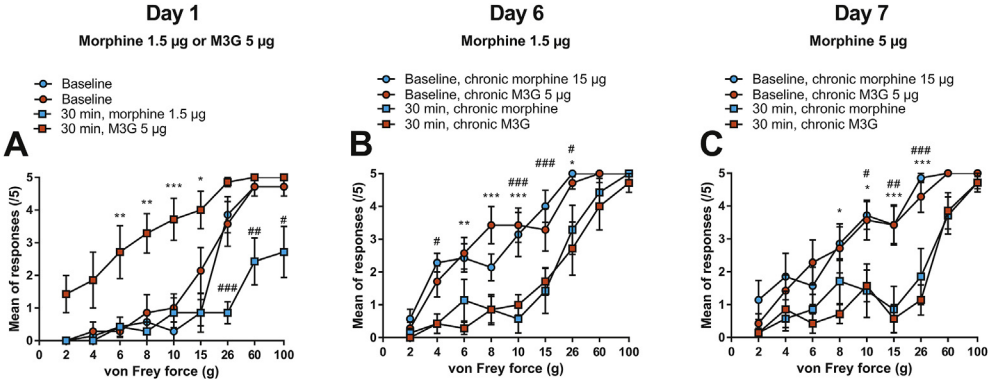

Fig. 2. The effect of acute and chronic morphine and M3G on tactile allodynia and mechanical nociception measured with von Frey filaments. Effects of acute administration of intrathecal morphine (1.5 μg) or morphine-3-glucuronide (M3G, 5 μg) on day 1 in naïve animals (A). Day 6 (B) and day 7 (C) show effects of intrathecal morphine (1.5 μg and 5 μg, respectively) in rats treated with chronic morphine (blue, 15 μg) or M3G (red, 5 μg). Results are presented as mean values ± S.E.M. Two-way ANOVA followed by Bonferroni's test. *P < 0.05, **P < 0.01 and ***P < 0.001 for chronically M3G treated group and #P < 0.05, ##P < 0.01 and ###P < 0.001 for chronically morphine treated group. n = 7.

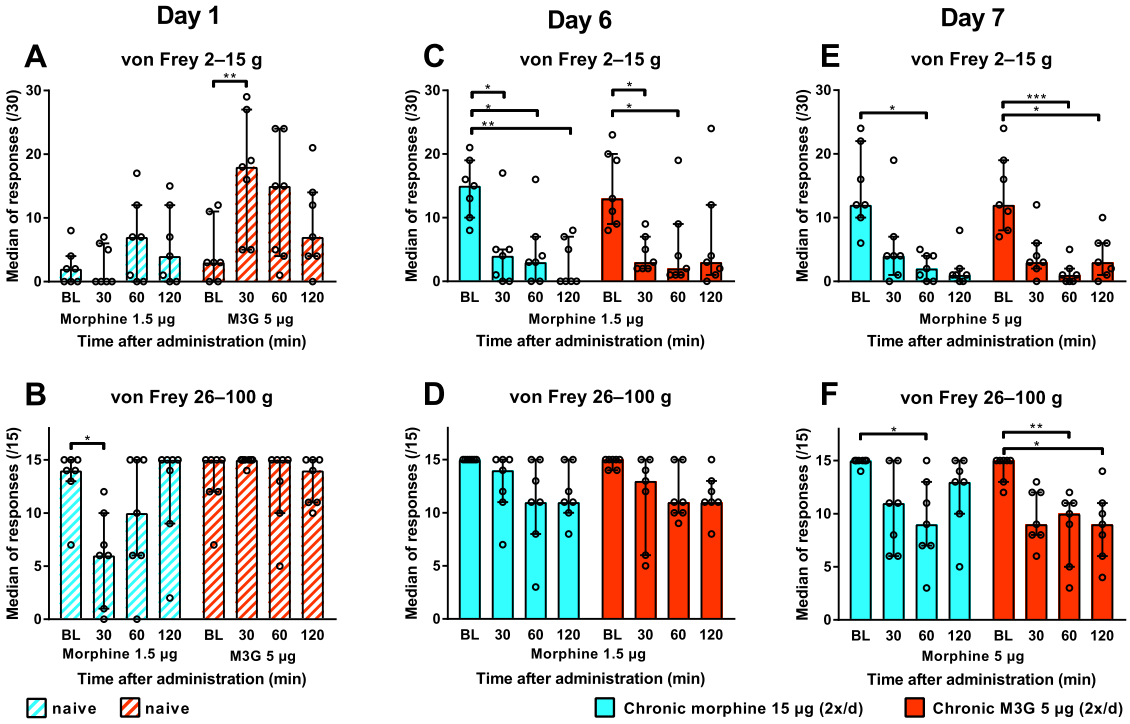

Fig. 3. Effects of acute administration of intrathecal morphine or morphine-3-glucuronide (M3G) in rats treated with chronic morphine or M3G on tactile allodynia and mechanical nociception, as measured with thin (2–15 g) and thick (26–100 g) von Frey filaments. Effects of intrathecal morphine (1.5 µg) or (M3G 5 µg) on the first day in naïve rats are shown for thin (A) and thick (B) filaments. Effects of morphine (1.5 µg) on the sixth day after chronic treatment were tested with thin (C) or thick (D) filaments, and on the seventh day after morphine (5 µg) with thin (E) or thick (F) filaments. Responses to different forces of the filaments are presented at baseline (BL) and 30–120 min after drug administration on days 1, 6 and 7. For the thin filaments, which were used to assess tactile allodynia, the sum of responses to 6 filaments (maximum 30) is shown, whereas for the thick filaments, which were used to assess morphine antinociception and development of tolerance, the maximum of responses was 15 (see Methods). Results are presented as median values ± interquartile range. *P < 0.05, **P < 0.01, and ***P < 0.001 (significance compared to BL values, Friedman test followed by Dunn's test for multiple comparisons). n = 7.

group are shown in Fig. 6E.

3.2.3. GFAP

The number of cells stained by the astrocyte marker GFAP or the area covered by GFAP positive staining per unit area of tissue was not significantly changed in the treatment groups, in either laminae I-II or lamina V (Fig. 7A–D). Representative staining images for each group are shown in Fig. 7E.

4. Discussion

M3G acutely sensitised animals to mechanical stimulation seen as an increased response rate to thin filaments but did not have an antinociceptive effect as studied with thick filaments. In contrast, acute morphine had an antinociceptive effect seen as a decreased response rate to thick filaments but it did not increase mechanical sensitivity. Although the acute effects were different, chronic treatment with both M3G and morphine caused similar development of antinociceptive tolerance to the morphine, tactile allodynia, and elevated levels of substance P in the nociceptive laminae of the spinal cord. The reduced effect of morphine on antinociception after M3G treatment, suggests development of antinociceptive cross-tolerance. Even though tolerance developed to the antinociceptive effects of morphine, it could still attenuate tactile allodynia after chronic M3G and morphine treatments.

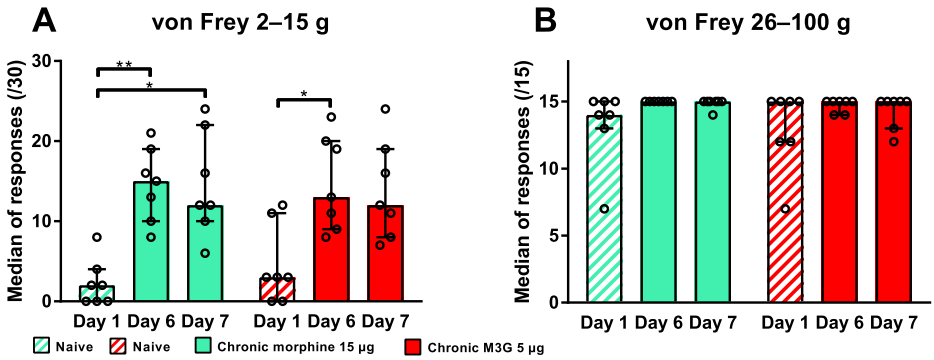

Fig. 4. Baseline (BL) values and effects of chronic administration of intrathecal morphine 15 µg and morphine-3-glucuronide (M3G) 5 µg on tactile allodynia (thin filaments) and mechanical nociception (thick filaments) on Days 1, 6, and 7 are shown. The median response rates to thin (2–15 g, A) and thick (26–100 g, B) von Frey filaments are shown. For the thin filaments, the sum of responses to 6 filaments (maximum responses 30) is shown, whereas for the thick filaments the maximum of responses was 15 (see Methods). Results are presented as median values ± interquartile range. *P < 0.05, **P < 0.01, and ***P < 0.001 (significance compared to BL values, Friedman test followed by Dunn's test for multiple comparisons). n = 7.

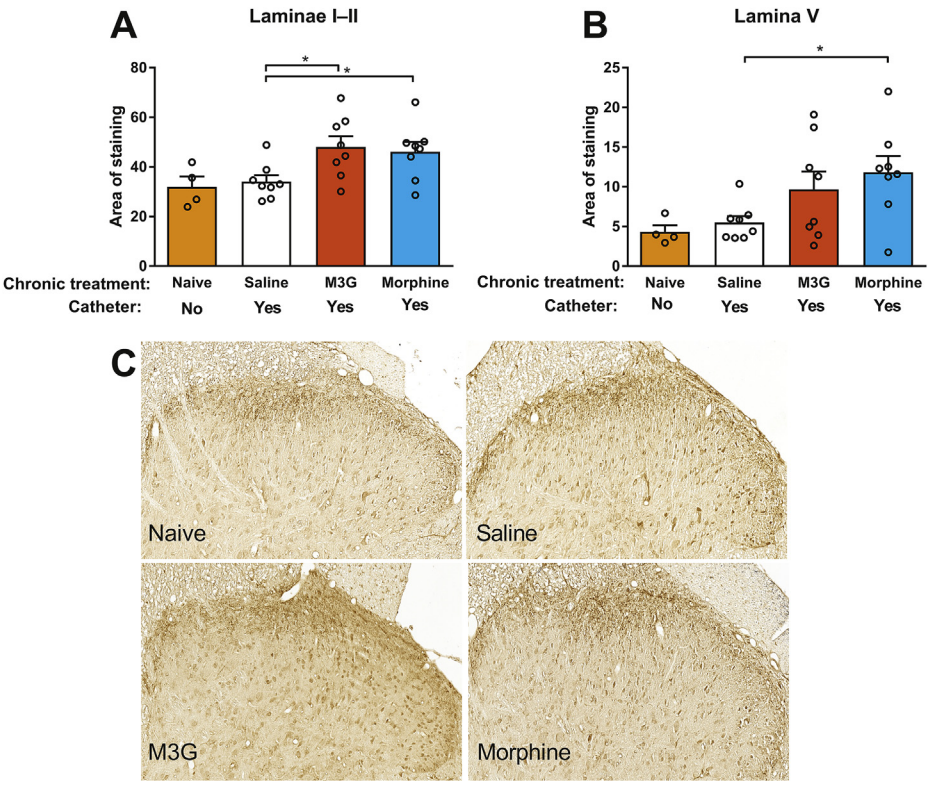

Fig. 5. Effects of 4 days of intrathecal morphine (15 μg) and morphine-3-glucuronide (M3G, 5 μg) administration on Substance P staining in nociceptive laminae (I–II, A and V, B) of the dorsal horn in the lumbar spinal cord. Non-catheterised animals (naïve) and catheterised animals that received saline were used as controls. Representative images for substance P-stained samples from the lumbar spinal cord dorsal horn are shown in C. Results are presented as mean values ± S.E.M. *P < 0.05, **P < 0.01, significance between naïve and saline compared with unpaired *t*-test and other groups except naïve by one-way ANOVA followed by Holm–Sidak's test. n = 8, except n = 4 in the non-catheterised naïve group. Values are formed from average of two spinal cord samples per animal.

These results suggest that M3G influences the development of allodynia and antinociceptive tolerance but do not mediate antinociception.

In line with previous studies (Gong et al., 1992; Komatsu et al., 2016; Lewis et al., 2010; Roeckel et al., 2017), acute intrathecal M3G administration caused tactile allodynia (Figs. 2A and 3A). In addition to this acute effect, both chronic M3G and morphine caused persistent tactile allodynia after a five-to six-day pretreatment (Fig. 3C and E; Fig. 4). In line with earlier studies (Angst and Clark, 2006; Ellis et al., 2016; Yaksh et al., 1986), chronic morphine induced tactile allodynia; our study adds that chronic M3G also induces tactile allodynia in a similar manner. Interestingly, acute morphine reversed the tactile allodynia caused by both chronic morphine and M3G. The development of sensitisation and reversal of it by acute morphine suggest that chronic M3G might partially modulate similar signaling pathways as chronic morphine does.

The response rate to thick filaments show that mechanical nociception after acute morphine (1.5 μg) was similar in both morphine- and M3G-treated groups (Fig. 3D). Also, antinociception was marginal after a 5-day chronic morphine or M3G treatment compared with Day 1, suggesting development of antinociceptive tolerance and cross-tolerance to morphine (Fig. 3D). Although M3G has been proposed to play a role in morphine-induced tolerance and hyperalgesia (Ekblom et al., 1993; Smith et al., 1990), we are not aware of any previous studies that would have shown the effect of chronic M3G pretreatment on the antinociceptive effect of acute morphine. These results suggest that in addition to sensitisation to mechanical stimuli, M3G can induce antinociceptive cross-tolerance to morphine.

Morphine is known to increase substance P levels and activate glial cells. In addition to having affinity to the μ receptor, morphine and M3G have also been suggested to activate microglia and induce opioid tolerance through the activation of toll-like receptor 4 (Due et al., 2012; Hutchinson et al., 2010; Lewis et al., 2010; Watkins et al., 2009). Therefore, we assessed the effects of M3G on the levels of substance P and glial cell markers. Substance P levels in the dorsal horn of the spinal cord were increased in a similar fashion after both chronic M3G and morphine treatments. Acutely administered morphine is known to inhibit the release of substance P in the dorsal horn of spinal cord, but the mechanism of the chronic opioid-induced increase of substance P (Bergstrom et al., 1984; Jokinen et al., 2018) levels is not known although elevated substance P is associated with neuroinflammation, pain, and opioid-induced hyperalgesia (King et al., 2005; Li and Clark, 2002; Ma et al., 2001). In our study, expression of the microglial marker IBA1 was not increased in the dorsal horn of the spinal cord compared with saline-treated animals. However, IBA1 expression was increased in all intrathecally catheterised groups when compared with intact animals. It is possible that the catheter-induced inflammatory reaction blunted the effects of the drugs. Catheter-induced gliosis in the spinal cord has been reported in one previous study (Mattioli et al., 2012). Because microglia is activated by substance P (Johnson et al., 2017; Zhu et al., 2014) and substance P immunoreactivity was increased by M3G in the present study, microglia may have a role in the observed antinociceptive cross-tolerance between M3G and morphine and sensitisation to the effect of chronic M3G.

The mechanisms behind M3G-induced mechanical sensitisation and antinociceptive cross-tolerance with morphine were not investigated in the present study. M3G was found not to cause hyperalgesia in μ receptor knockout animals, supporting the role of the μ receptor as mediator of the pronociceptive effects of M3G. (Roeckel et al., 2017). The K_i values for M3G and morphine at the μ receptor are 360 nM and 1.8 nM, respectively, indicating low affinity of M3G at the μ receptor (Mignat et al., 1995). However, M3G concentrations (719 nM) clearly exceeding this K_i have been measured in the cerebrospinal fluid of cancer patients receiving high-dose systemic morphine (Goucke et al., 1994). M3G is very hydrophilic and resides mostly in the extracellular fluid (Okura et al., 2003). Although cerebrospinal fluid concentrations are not a direct measure of brain or spinal cord extracellular fluid concentrations, it is possible that after a long-term high-dose morphine treatment M3G may have clinically significant effects mediated by the μ receptor.

Opioid tolerance has been linked to desensitisation and down-regulation of opioid receptors, activation of the β-arrestin 2 pathway, cellular adaptive changes such as upregulation of adenylyl cyclase and

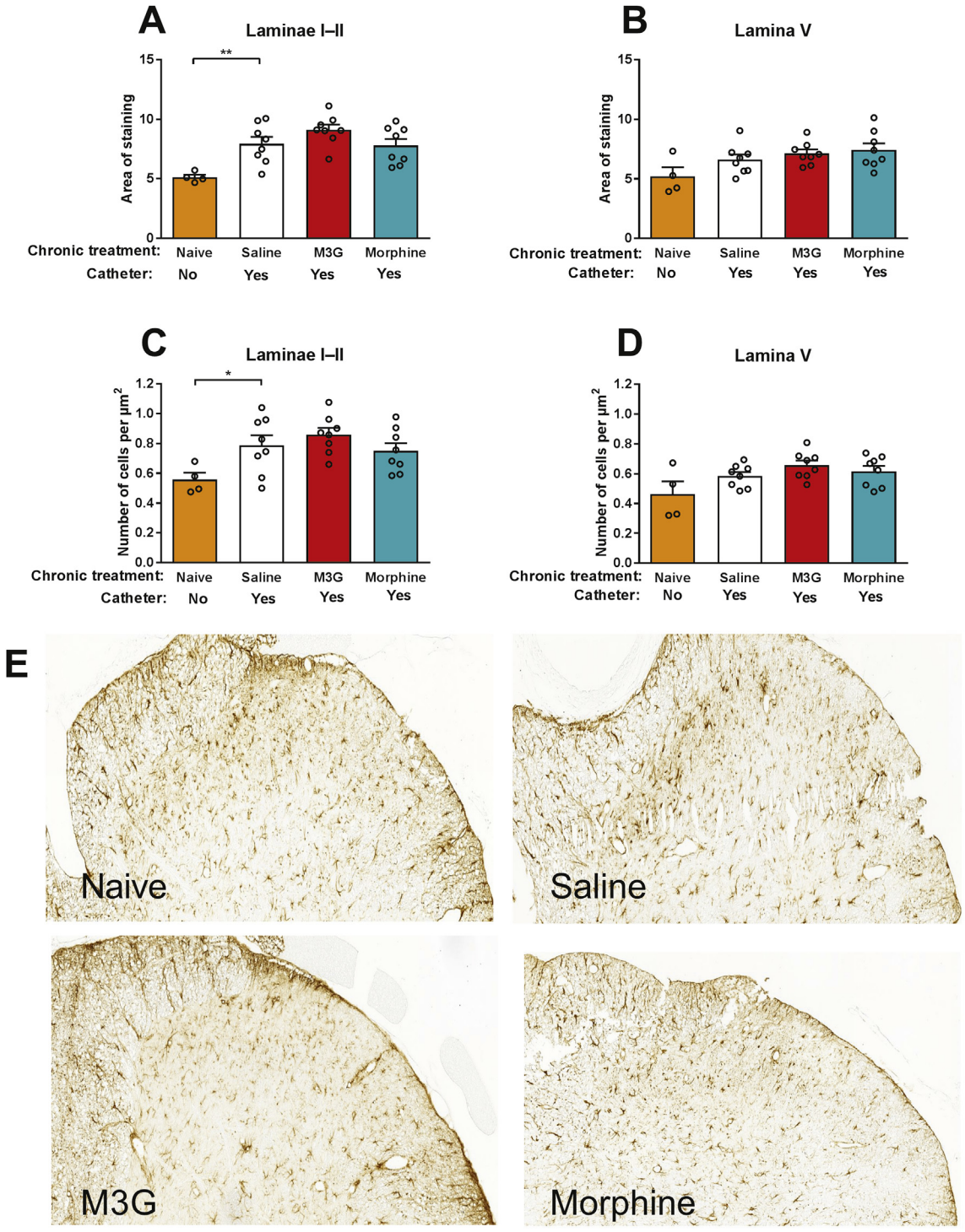

Fig. 6. Effects of 4 days of intrathecal morphine (15 µg) and morphine-3-glucuronide (M3G, 5 µg) administration on the microglial marker IBA1 in nociceptive laminae (I–II and V) of spinal cord. The relative area of staining is shown in A–B and the number of IBA1-positive cells per μm^2 shown in C–D. Non-catheterised animals (naïve) and catheterised animals that received saline were used as controls. Representative images for IBA1-stained samples from the lumbar spinal cord dorsal horn are shown in (E). Results are presented as mean values ± S.E.M. *P < 0.05, **P < 0.01, significance between naïve and saline compared with unpaired *t*-test and other groups except naïve by one-way ANOVA followed by Holm–Sidak's test. n = 8, except n = 4 in the non-catheterised naïve group. Two samples per animal were analysed and the average used.

changes in MAPK signaling (Al-Hasani and Bruchas, 2011; Bohn et al., 2000; Rivat and Ballantyne, 2016). *In vitro*, M3G causes weak G_i activation but does not activate the β-arrestin 2 pathway suggesting that M3G activates slightly different signaling pathways compared with morphine (Roeckel et al., 2017). However, because M3G does not recruit β-arrestin 2, an important signaling pathway for the development of opioid tolerance (Bohn et al., 2000), the biased agonist properties of M3G most likely do not explain antinociceptive cross-tolerance between M3G and morphine. Another explanation could be that similar to morphine, M3G activates the 6-transmembrane subtype of µ receptors (Oladosu et al., 2015) or toll-like receptor 4 (Due et al., 2012). Detailed studies on the mechanism of M3G-induced opioid tolerance and

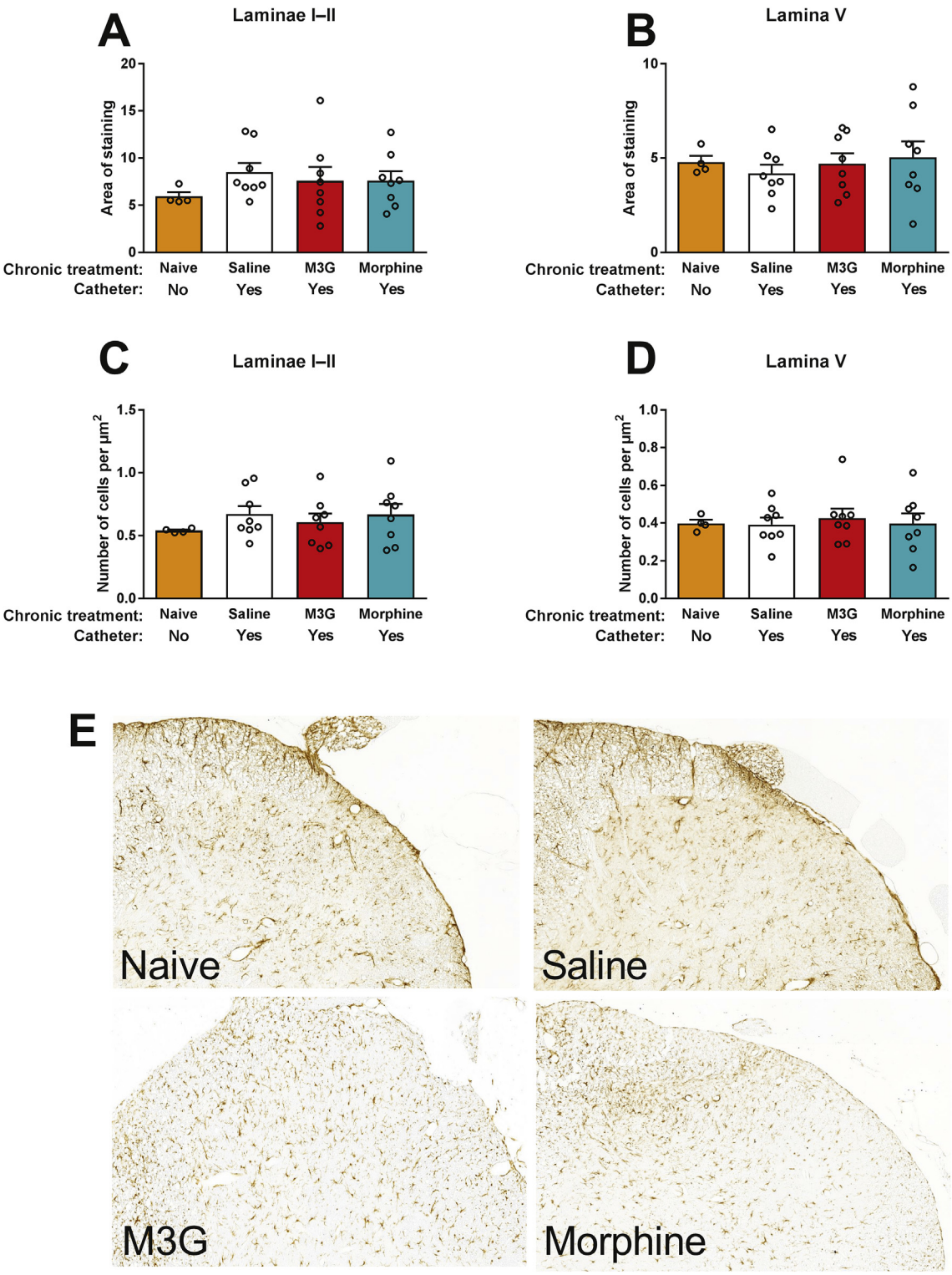

Fig. 7. Effects of 4 days of intrathecal morphine (15 µg) and morphine-3-glucuronide (M3G, 5 µg) administration on the astrocyte marker GFAP in nociceptive laminae (I–II and V) of spinal cord. The relative area of staining is shown in A–B and the number of GFAP-positive cells per µm^2 shown in C–D. Non-catheterised animals (naïve) and catheterised animals that received saline were used as controls. Representative images for GFAP-stained samples from the lumbar spinal cord dorsal horn are shown in (E). Results are presented as mean values ± S.E.M., statistical comparison between naïve and saline compared with unpaired *t*-test and other groups except naïve by one-way ANOVA followed by Holm–Sidak's test showed no significance. n = 8, except n = 4 in the non-catheterised naïve group. Two samples per animal were analysed and the average used.

hyperalgesia are needed.

In conclusion, chronic intrathecal M3G treatment leads to persistent tactile allodynia and antinociceptive cross-tolerance to morphine in a similar way as chronic intrathecal morphine treatment does. In addition, M3G-induced mechanical sensitisation could be reversed by morphine. M3G increased spinal cord levels of substance P suggesting that the excitatory effect of substance P may have a role in M3G-induced allodynia.

Funding

This research has received funding from the European Union Seventh Framework Programme (FP7/2007–2013) under grant agreement no 602919; European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 798944; The Finnish Medical Society (Finska Läkaresällskapet), Helsinki, Finland and University of Helsinki 375th Anniversary Grant, University of Helsinki.

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Declaration of competing interest

E.K. is a member of advisory boards (Orion Pharma, Espoo, Finland, and Pierre Fabre, Toulouse, France). Other authors have no conflicts of interest to be reported.

Acknowledgments

National Institution of Drug Abuse, Bethesda, MD, USA is acknowledged for the kind gift of morphine-3-glucuronide. Jenni Montonen, Laboratory of Molecular Neuroscience, Institute of Biotechnology, is acknowledged for expert assistance. Les Hearn is acknowledged for scientific proofreading (les_hearn@yahoo.co.uk).

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